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Juan Du

Shaoyun Lu

Maofeng Chai

Chuanen Zhou

Liang Sun

*See next page for additional authors*

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## Authors

Juan Du, Shaoyun Lu, Maofeng Chai, Chuanen Zhou, Liang Sun, Yuhong Tang, Jaydeep Kolape, Zhaozhu Wen, Marjan Behzadirad, Tianxiu Zhong, Juan Sun, Yunwei Zhang, and Zeng-Yu Wang

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# Functional characterization of *PETIOLULE-LIKE PULVINUS (PLP)* gene in abscission zone development in *Medicago truncatula* and its application to genetic improvement of alfalfa

Juan Du<sup>1,2,3</sup>, Shaoyun Lu<sup>4</sup>, Maofeng Chai<sup>1,5</sup>, Chuanen Zhou<sup>6</sup>, Liang Sun<sup>1</sup>, Yuhong Tang<sup>1</sup>, Jin Nakashima<sup>1</sup>, Jaydeep Kolape<sup>1,7</sup> , Zhaozhu Wen<sup>1,8</sup>, Marjan Behzadizad<sup>2</sup>, Tianxiu Zhong<sup>9</sup>, Juan Sun<sup>5</sup>, Yunwei Zhang<sup>3,\*</sup> and Zeng-Yu Wang<sup>1,5,\*</sup> 

<sup>1</sup>Noble Research Institute, Ardmore, OK, USA

<sup>2</sup>Institute for Agricultural Biosciences, Oklahoma State University, Ardmore, OK, USA

<sup>3</sup>College of Grassland Science and Technology, China Agricultural University, Beijing, China

<sup>4</sup>College of Life Sciences, South China Agricultural University, Guangzhou, China

<sup>5</sup>Grassland Agri-Husbandry Research Center, College of Grassland Science, Qingdao Agricultural University, Qingdao, China

<sup>6</sup>School of Life Science, Shandong University, Qingdao, China

<sup>7</sup>Morrison Microscopy Core Research Facility, Center for Biotechnology, University of Nebraska-Lincoln, NE, USA

<sup>8</sup>College of Agriculture, Hunan Agricultural University, Hunan, China

<sup>9</sup>College of Forestry and Landscape Architecture, South China Agricultural University, Guangzhou, China

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\*Correspondence (Tel: 86-18866267950; e-

mail: zywang@qau.edu.cn (Z.Y.W.) or Tel:

86-10-62733572; e-mail:

zwang98\_99@yahoo.com (Y.Z.)

## Summary

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops throughout the world. Maximizing leaf retention during the haymaking process is critical for achieving superior hay quality and maintaining biomass yield. Leaf abscission process affects leaf retention. Previous studies have largely focused on the molecular mechanisms of floral organ, pedicel and seed abscission but scarcely touched on leaf and petiole abscission. This study focuses on leaf and petiole abscission in the model legume *Medicago truncatula* and its closely related commercial species alfalfa. By analysing the *petiolule-like pulvinus (plp)* mutant in *M. truncatula* at phenotypic level (breakstrength and shaking assays), microscopic level (scanning electron microscopy and cross-sectional analyses) and molecular level (expression level and expression pattern analyses), we discovered that the loss of function of *PLP* leads to an absence of abscission zone (AZ) formation and *PLP* plays an important role in leaflet and petiole AZ differentiation. Microarray analysis indicated that *PLP* affects abscission process through modulating genes involved in hormonal homeostasis, cell wall remodelling and degradation. Detailed analyses led us to propose a functional model of *PLP* in regulating leaflet and petiole abscission. Furthermore, we cloned the *PLP* gene (*MsPLP*) from alfalfa and produced RNAi transgenic alfalfa plants to down-regulate the endogenous *MsPLP*. Down-regulation of *MsPLP* results in altered pulvinus structure with increased leaflet breakstrength, thus offering a new approach to decrease leaf loss during alfalfa haymaking process.

**Keywords:** abscission, alfalfa, forage improvement, *Medicago truncatula*, *Medicago sativa*, *PETIOLULE-LIKE PULVINUS (PLP)*.

## Introduction

Alfalfa (*Medicago sativa* L.) is one of the most widely cultivated forage species in the world and the fourth most valuable crop in the United States after corn, soybean and wheat (Zhang *et al.*, 2005). Alfalfa has high biomass yield, excellent nutritional quality, wide adaptation and nitrogen fixation ability (Putnam *et al.*, 2008). In the United States, it is estimated that more than 95% alfalfa is used for hay production. Leaf loss during the alfalfa haymaking process is considerable, which is in the range of 0.84% to 15.5% of hay dry matter (Pitt, 1990). Based on alfalfa production and price data from USDA National Agricultural Statistics Service (2018 data), haymaking process led to 0.5–8.6 million tons of leaf loss and economic loss of 0.07 to 1.16 billion

dollars in the United States per year. The current methods to decrease mechanical leaf loss include optimization of alfalfa harvesting conditions, mechanical or chemical conditioning and utilization of advanced machinery (Summers & Idowu *et al.*, 2013; Putnam *et al.*, 2008). However, such management practice requires extensive experience and is difficult to control due to unpredictable weather conditions. Based on our observation during alfalfa hay making process, over 95% leaf loss occurred specifically at the leaflet and petiole pulvinus region, which is a joint-like thickening located at the base of the petiole or petiolule. The weakness of leaflet and petiole pulvinus region might due to the pulvinus structure or the progressive degradation of middle lamella under abscission process at that region. Therefore, strengthening pulvinus structure and manipulating leaflet and

petiole abscission are promising targets in alfalfa genetic improvement.

Abscission is a developmental process allowing a plant to shed its unwanted organs in response to various developmental, hormonal or environmental cues (Addicott, 1982; Estornell *et al.*, 2013; Roberts *et al.*, 2002). Abscission occurs at functionally specialized cell layers, called abscission zone (AZ), which is developed at the junction between the leaving organ and the main plant body. AZ is composed of a few layers of small and dense cytoplasm cells (Addicott, 1982; Osborne and Morgan, 1989; Sexton and Roberts, 1982). The abscission process has been divided into four major phases according to studies of floral organ abscission in *Arabidopsis thaliana*: (i) abscission zone differentiation; (ii) acquisition of the competence to respond to abscission signals; (iii) activation of abscission; and (iv) protective layer formation (Estornell *et al.*, 2013; Kim, 2014; Patharkar and Walker, 2018; Patterson, 2001; Taylor and Whitelaw, 2001). All of these steps are important for the fate of organs that will shed.

The prerequisite of abscission is AZ differentiation; a set of genes participating in this process has been identified in *Arabidopsis*, tomato and rice (Lewis *et al.*, 2006). In *Arabidopsis*, *BLADE-ON-PETIOLE1* (*BOP1*)/*BOP2*, *ARABIDOPSIS THALIANA* *HOMEBOX GENE1* (*ATH1*), *BREVIPELCELLUS1* *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1* (*BPKNAT1*) and *ASYMMETRIC LEAVES 1* (*AS1*) are critical for floral organ AZ differentiation (Gómez-Mena and Sablowski, 2008; Gubert *et al.*, 2014; McKim *et al.*, 2008; Shi *et al.*, 2011). Several genes regulating pedicel AZ differentiation have been identified, including *JOINTLESS*, *LATERAL SUPPRESSOR* (*LS*), *MACROCALYX* (*MC*) and *SLMBP21* (Liu *et al.*, 2014; Mao *et al.*, 2000; Nakano *et al.*, 2012; Schumacher *et al.*, 1999). In rice, *SHATTERING4* (*SH4*), *qSH1*, *OsSH1* and *SHATTERING ABORTION 1* (*SHAT1*) function as positive regulators to modulate pedicel AZ development, while *OsCPL1* represses pedicel abscission zone differentiation (Ji *et al.*, 2010; Konishi *et al.*, 2006; Li *et al.*, 2006; Lin *et al.*, 2012; Zhou *et al.*, 2012b).

After AZ is differentiated, the AZ cells will be staying at a quiescent state, until they perceive abscission-promoting signals to initiate the abscission process (Nakano *et al.*, 2013). Abscission signals will activate the expression of genes such as cell wall hydrolytic enzymes (e.g. polygalacturonases) to act on structural polysaccharides leading to the hydrolysis of the middle lamella and cell walls of the AZ cells (Estornell *et al.*, 2013). Both ethylene and auxin (IAA) were previously proposed as important signals for abscission activation (Taylor and Whitelaw, 2001). However, ethylene-insensitive mutants *ethylene resistant 1-1* (*etr1-1*) and *ethylene-insensitive 2* (*ein2*) showed delayed instead of abolished floral organ abscission, indicating that ethylene is not critical for abscission, and thus, auxin has been proposed as a key player in the regulation of abscission process (Patterson and Bleecker, 2004; Taylor and Whitelaw, 2001). Auxin response factors (ARFs) are transcription factors that mediate responses to auxin. In *Arabidopsis*, *arf2* mutant delayed floral organ abscission, while *arf1* enhanced *arf2* phenotype (Okushima *et al.*, 2005). The mutation of *NPH4/ARF7* and *ARF19* enhanced *arf2* phenotype (Ellis *et al.*, 2005). It has been shown that indoleacetic acid (IAA) signalling is a prerequisite for floral organ shedding in *Arabidopsis* (Basu *et al.*, 2013). Microarray analysis of gene alterations in response to auxin depletion in tomato and *Mirabilis jalapa* revealed that acquisition of ethylene sensitivity in leaf and flower AZ is associated with differential expression of auxin-related genes (Meir *et al.*, 2006; Meir *et al.*, 2010). In tomato, *KD1*, a *KNOTTED1-LIKE* *HOMEBOX* (*KNOX*) family gene, was identified

to modulate pedicel and petiole abscission by regulating genes involved in the auxin signalling pathway (Ma *et al.*, 2015). However, the transcriptional regulation of hormonal homeostasis in the leaf AZ is still unclear.

To date, a wealth of valuable information has been accumulated based on the studies of floral organ abscission in *Arabidopsis*, pedicel abscission in tomato and seed shattering in rice. However, our understanding of molecular mechanisms on leaf abscission is still limited. Recently, it has been revealed that both drought- and pathogen-triggered cauline leaf abscission in *Arabidopsis* might be regulated through salicylic acid signalling (Patharkar and Walker, 2016; Patharkar *et al.*, 2017). *HAESA/HAESA-like 2* (*HAE/HSL2*), *INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*), *NEVERSHED* (*NEV*) and *MAPK KINASE4/5* (*MKK4/5*) are required for drought-induced leaf abscission (Patharkar and Walker, 2016). *HAESA/HAESA-like 2* (*HAE/HSL2*), *IDA* and *NEVERSHED* (*NEV*) are required for the initiation of pathogen-triggered leaf abscission (Patharkar *et al.*, 2017). *BOP1/BOP2* in *Arabidopsis* and its ortholog *NODULE ROOT* (*NOOT*) in *Medicago truncatula* have been shown to be involved in leaf abscission zone formation (Couzigou *et al.*, 2016; McKim *et al.*, 2008). Recent transcriptome studies of leaf abscission have provided insights into the regulatory mechanism of abscission (Kim *et al.*, 2016; Li *et al.*, 2017; Li *et al.*, 2016; Liao *et al.*, 2016a, 2016b, 2016c).

As a model plant for legume species, *Medicago truncatula* is also suitable for leaf abscission study. It has compound leaves and its leaflets and petioles abscise at the senescing stage at the base of pulvinus. Over the years, with the development of genomic resources, particularly the generation of large-scale insertional mutagenesis (Tadege *et al.*, 2005) and the sequencing of the *M. truncatula* genome (Young *et al.*, 2011), many genes with novel functions have been identified (Chai *et al.*, 2016; Kang *et al.*, 2016; Zhao *et al.*, 2019; Zhou *et al.*, 2019). Leguminous plants open their leaves during the day and close them at night, and this kind of nyctinastic movement is induced by volume change in motor cells in the pulvinus (Ueda and Nakamura, 2007). The *PETIOLULE-LIKE PULVINUS* (*PLP*) gene was identified by forward screening from the retrotransposon-tagged mutant population of *M. truncatula*; the loss of function of *PLP* caused the alteration of pulvini to petiolules and affected the nyctinastic movement of leaflets (Zhou *et al.*, 2012a). In this study, we report novel findings that the loss of function of *PLP* results in an absence of AZ formation and *PLP* plays an important role in leaflet and petiole AZ differentiation, thus providing new information on molecular mechanism of leaf and petiole abscission. Furthermore, down-regulation of the *PLP* gene (*MsPLP*) in alfalfa results in the alteration of pulvinus to petiolule-like structure with increased leaflet breakstrength, offering a new approach to minimize leaf loss during alfalfa haymaking process.

## Results

### Mutation in *PLP* abolishes shedding of leaflet and petiole in *M. truncatula*

The *PLP* gene has one exon with 579 nucleotides containing a LOB domain (Zhou *et al.*, 2012a). To characterize the role of *PLP* in leaflet and leaf abscission, three alleles *plp-1* (a *Tnt1* inserted after base 178), *plp-2* (a *Tnt1* inserted after base 130) and *plp-4* (a *Mere1* inserted after base 246) were used for analysis (Figure 1a). The loss of function of *PLP* altered the leaflet pulvini into petiolule-like structures (Figure S1a-d) and the petiole pulvini into petiole-like structures (Figure S1e-h).

In wild-type *M. truncatula*, organ detachment started with leaflet at the base of pulvinus (Figure 1b,d) and then followed by petiole at the stem–petiole junction (Figure 1f,h). Leaflet and petiole started to shed at the leaf age of 35 days (67% remaining) and 37 days (87% remaining), respectively (Table S1). The percentage of plants with remaining leaves and petioles decreases with the increase of leaf ages until no leaflets and petioles were left on the plant, when the leaf age reaches 43 and 45 days (Table S1). In contrast, both leaflets and petioles of the *plp* mutant remain attached to the plant even after 45 days (Table S1). In *plp* mutants, shedding of leaflet and petiole was abolished even when the whole plant was dead.

The abscission defects of *plp* were further characterized by shaking assay. After shaking vigorously for 2 min, almost all the senescing leaves of wild type shed, while for *plp* mutants, most petioles and leaflets were still attached to the plant body (Figure S2a). Markedly reduced leaf shed was observed for *plp* with respect to wild-type plants (Figure S2b–g). The weight of dropped leaves of wild type was 5 times more than that of *plp* (Figure S2h). The shaking assay further confirmed that *plp* has a defect in leaflet and petiole abscission at senescing stage.

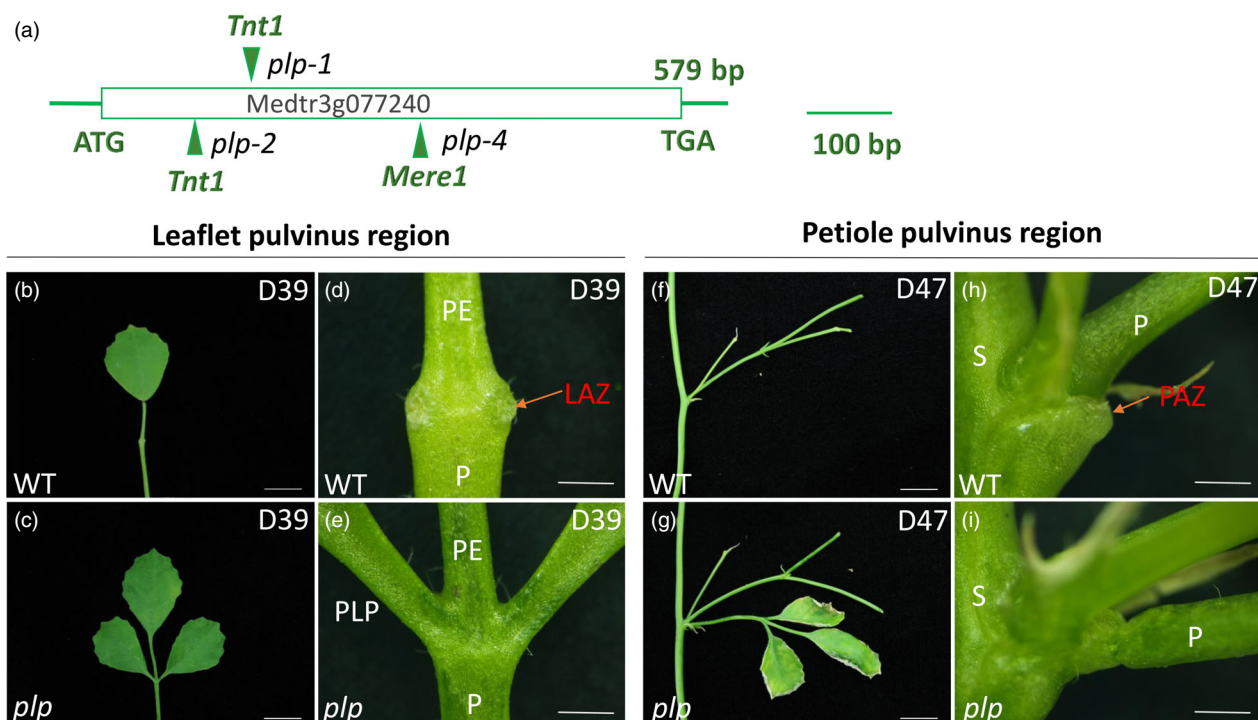
In order to further characterize the leaflet and petiole abscission defects of *plp*, a breakstrength assay was performed. The wild-type leaflet and petiole breakstrength gradually decreased after 33 days old and 35 days old, respectively, indicating a progressive degradation of the middle lamellae in leaflet and petiole abscission zone (Figure 2). However, leaflet and petiole

breakstrength of *plp* showed no obvious change from 33- to 39-day-old leaf and 35- to 39-day-old leaf (Figure 2). After 39 days, the leaflet and petiole breakstrength of *plp* gradually decreased with the increase of leaf ages (Figure 2). In all cases, both leaflet breakstrength and petiole breakstrength of *plp* mutants were significantly higher than wild type (Figure 2).

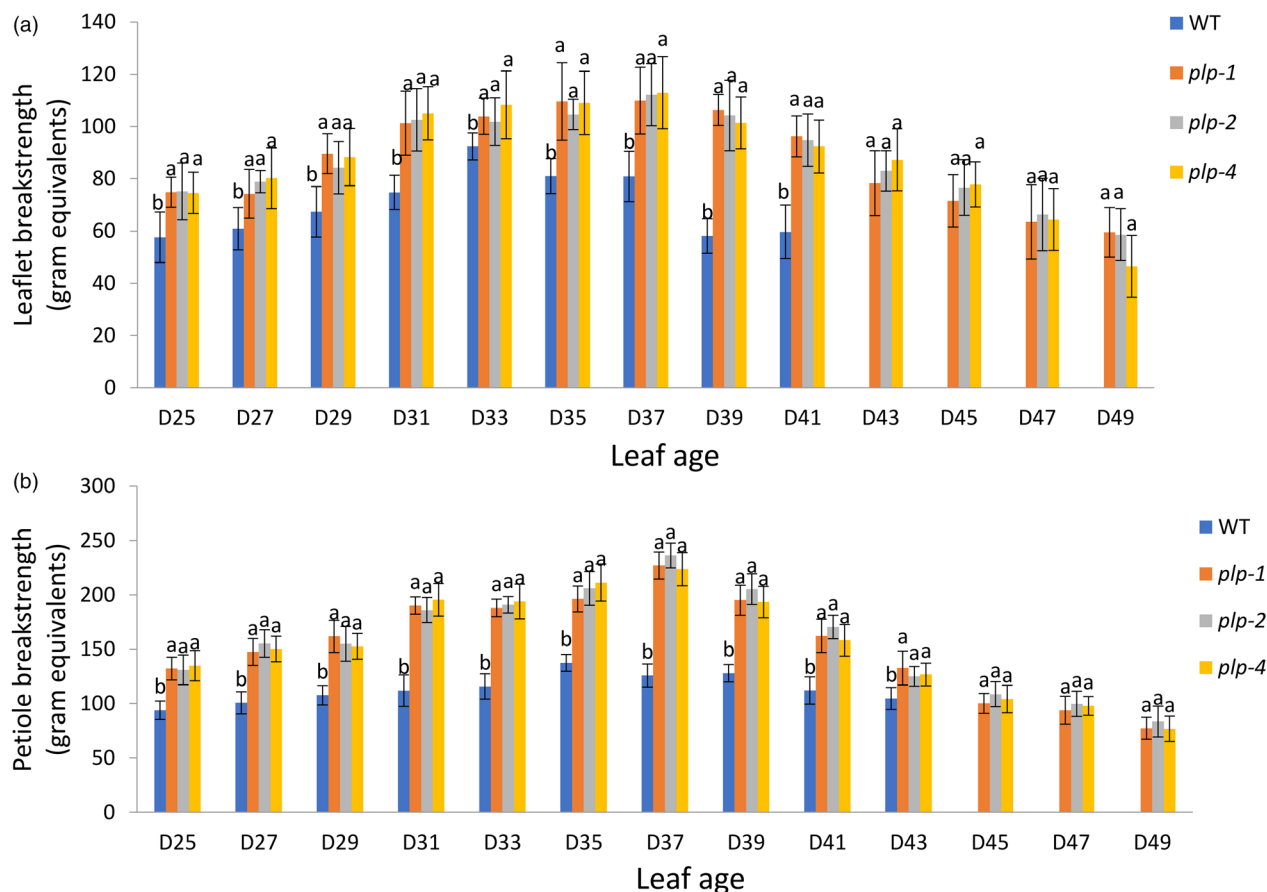
#### PLP plays an important role in controlling leaflet abscission zone (LAZ) and petiole abscission zone (PAZ) differentiation

To further determine whether normal LAZ and PAZ were developed in *plp*, we used scanning electron microscopy (SEM) to examine the leaflet pulvinus–petiolule boundary and petiole pulvinus–stem boundary of wild type and *plp* mutant (Figure 3). In wild type, scars were left after manual removal of leaflet and petiole from the plant. Ruptured cells were observed upon removal of 31-day-old leaflet and 35-day-old leaflet at the fracture plane (Figure 3b,c). Owing to the weakening of the middle lamellae, removal of 39-day-old leaflet only caused very few cell ruptures (Figure 3d). Leaflets were already abscised at 43 days and 47 days, and there was a protective layer of rounded cells formed at the leaflet abscission zone (Figure 3e,f). By contrast, the leaflet fracture surface of *plp* mutant sample showed no evidence of AZ activation at any age and cells ruptured during leaflet removal (Figure 3g–k).

Broken cells were observed upon removal of petiole (from 31 to 43 days old) in wild type (Figure 3l–o). Due to the abscission of



**Figure 1** Petiolule-like pulvinus (*plp*) mutant of *M. truncatula* showing defects in leaflet and petiole abscission. (a) Schematic representation of the gene structure of *PLP*. The arrows indicate the location of *Tnt1* and *Mere1* retrotransposons in the *plp* alleles. (b, c) Senescing leaf (39-day-old) of wild type and *plp* mutant. Wild-type leaflet shed at this stage. (d, e) Close-up view of leaflet base at leaflet abscission zone (LAZ) in wild type and *plp* mutant. Red arrow indicates the LAZ in wild type. The leaflet has already abscised from wild type, with the AZ left at the base of pulvinus, while for *plp* mutant, leaflet is still attached to petiole. (f, g) Senescing leaf (47-day-old) with petiole abscission zone. Wild-type leaf shed at this stage. (h, i) Close-up view of petiole abscission zone of wild type and *plp* mutant. Red arrow indicates petiole abscission zone (PAZ). The petiole of wild type has already abscised from stem with the PAZ left at the base of pulvinus, while *plp* petiole is still attached to stem, although the leaflets are nearly dry. PE, petiolule; P, petiole; PLP: petiolule-like pulvinus; S, stem. D39, 39-day-old; D47, 47-day-old. Scale bars: (b, c, f, g) 1 cm; (d, e, h, i) 1 mm. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Figure 2** Leaflet and petiole breakstrengths of *plp* and wild type at different stages in *M. truncatula*. (a) Leaflet breakstrength measurements of *plp* and wild type at different stages. Wild-type leaflets have already dropped when leaf age reached 43 days. (b) Petiole breakstrength measurements of *plp* and wild type at different stages. Wild-type petiole has already dropped when leaf age reached 45 days. D25, 25-day-old leaf; D27, 27-day-old leaf; ... D49, 49-day-old leaf. Error bars represent SD ( $n = 8$ ). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

petiole in 47-day-old leaf, the petiole fracture surface of wild type showed a layer composed of spherical elongated cells (Figure 3p). However, in *plp* mutant, ruptured cells at fractured plane were observed at all leaf ages (Figure 3q-u).

Phenotype characterization and SEM analysis revealed the important role of PLP in LAZ and PAZ development. To determine more precisely the differences in cellular morphology between wild type and *plp*, longitudinal sections of leaflet and petiole pulvinus region at 25 and 35 days were examined and compared (Figure 4). The 25-day-old leaf (mature leaf) represents the stage before abscission process occurs, whereas the 35-day-old leaf (senescing leaf) represents the stage the abscission process was undergoing at both leaflet and petiole abscission zones based on the observation in Table S1. In wild type, a layer of small cytoplasmic dense cells at the junction between pulvinus and petiolule was differentiated at 35 days (Figure 4e, red arrow), and the morphological differences were apparent when compared with the 25-day-old section (Figure 4c). The edge of the pulvinus and petiolule junction was detached due to the dissolvment of middle lamella of LAZ cells (Figure 4e). Unlike the wild-type control, no differentiated small cells were detected in the *plp* region which is marked by red asterisk indicative of the region corresponding to wild-type LAZ (Figure 4f). Compared with 25-day-old section at petiole pulvinus region, cytoplasmic dense AZ cell layers marked by red arrow were visible between

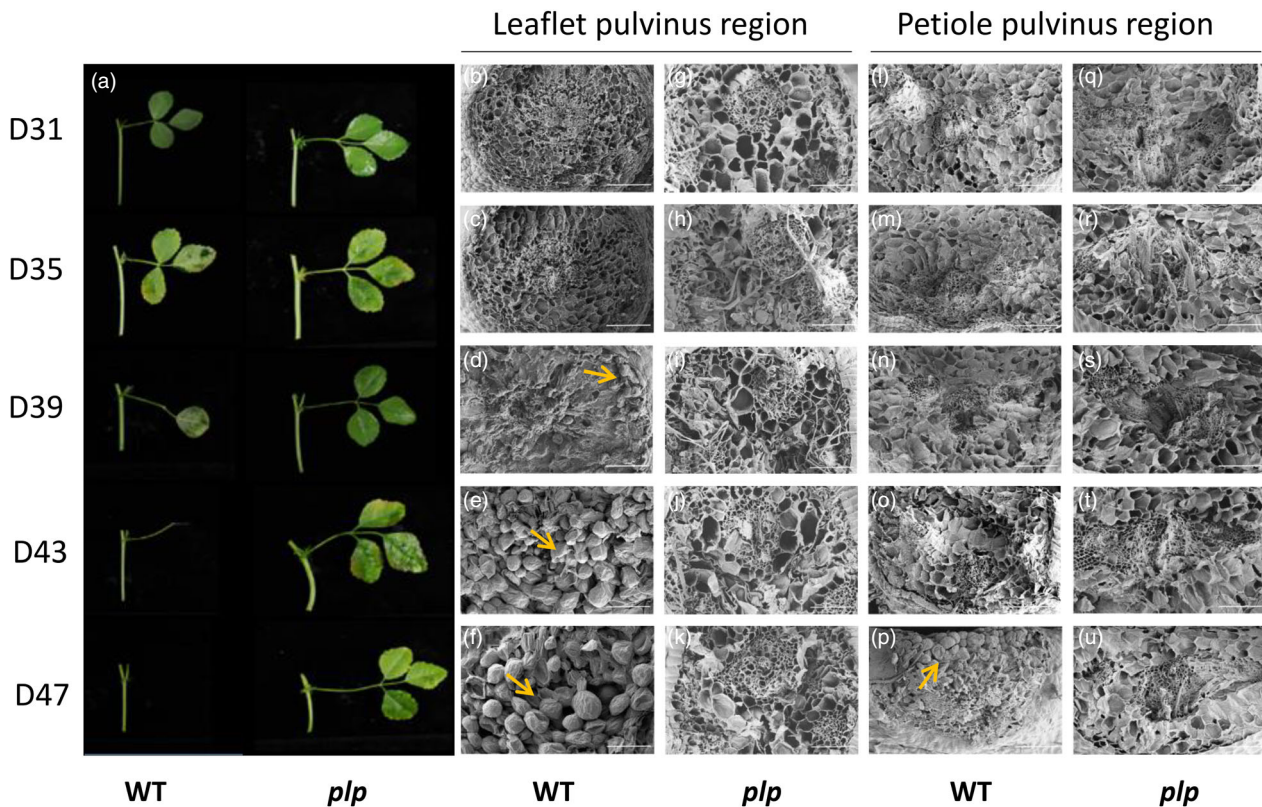
petiole pulvinus and stem in wild type at 35 days (Figure 4k). However, *plp* did not show such a structure differentiated at the corresponding location marked by a red asterisk at 35-day-old leaf (Figure 4l). Therefore, the distinct cell layers that differentiated at the junctions between pulvinus and petiolule and between petiole and stem were absent in *plp*.

#### PLP expression pattern during the leaf senescing process

The expression pattern of *MtPLP* in wild-type plants was analysed by *M. truncatula* Gene Expression Atlas (Benedito *et al.*, 2008). The expression of *MtPLP* was detected in almost all the organs at various developmental stages, with a relatively high expression level in vegetative buds and nodules and relatively low levels in seed and leaf (Figure S3).

To determine whether there is a correlation between the *PLP* expression pattern and leaflet and petiole abscission, we analysed transgenic *M. truncatula* plants that expressed GUS under the control of *PLP* promoter. We examined GUS expression during the time course of abscission in three stages as shown in Table S2: stage I, prior to organ separation; stage II, during abscission; and stage III, after abscission when the remaining cells form protective scar tissue. The expression of the *proPLP::GUS* construct was observed in both leaflet and petiole pulvinus regions and at the base of leaflet–petiolule junction and stem–petiole junction (Figure 5a,e). Prior to organ abscission, the expression of *PLP*





**Figure 3** Abscission phenotype and scanning electron micrographs of leaflet and petiole abscission zones in *M. truncatula*. (a) Leaves of wild type and *plp* at different ages. In wild-type plants, 39-day-old leaflet started to abscise from the petiolule and petiole of 47-day-old leaf had already abscised from stem, while in *plp* plants, leaflets and petiole did not abscise. (b–f) Leaflet fracture planes of wild type showing progression from broken cells (31-, 35- and 39-day-old leaflets) to rounded AZ cells (43- and 47-day-old leaflets). (g–k) Leaflet fracture planes of *plp* mutant showing broken cells (all ages). (l–p) Petiole fracture planes of wild type showing progression from broken cells (31-, 35-, 39- and 43-day-old leaves) to rounded AZ cells (47-day-old leaves). (q–u) Petiole fracture planes of *plp* mutant showing broken cells (all ages). D31, 31-day-old leaf; D35, 35-day-old leaf; D39, 39-day-old leaf; D43, 43-day-old leaf; D47, 47-day-old leaf. Scale bars: 100  $\mu$ m. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

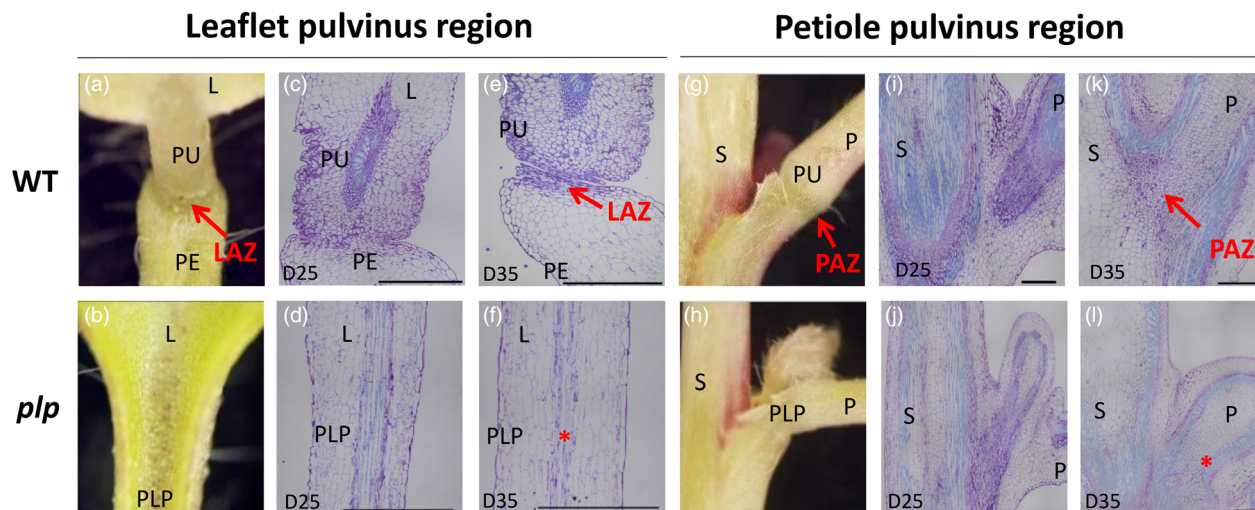
was restricted to the abscission zones, in which the leaflet and petiole had not detached (Figure 5b,f). During abscission, a strong signal was detected at the base of leaflet and petiole pulvinus region (Figure 5c,g). After abscission, at the AZ scars following leaflet and petiole detachment, no GUS signal was detected (Figure 5d,h). Taken together, the *proPLP::GUS* expression pattern showed that *PLP* is strongly expressed in the abscission zone prior to abscission and during abscission but not observed after abscission, confirming that *PLP* contributes to AZ differentiation.

#### Gene expression analysis identified genes regulated by *PLP* in the abscission process

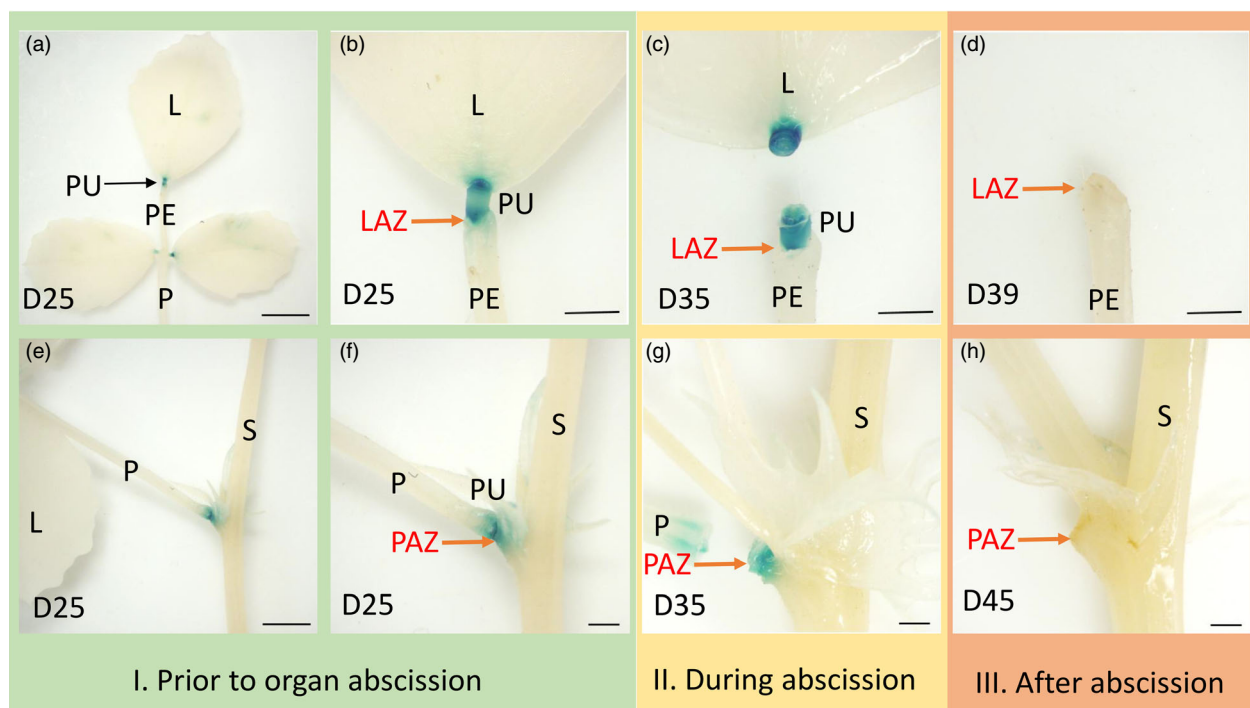
To explore the transcriptional mechanisms underlying the loss of function of AZ development in *plp* mutants, gene expression in 25-day-old leaflet AZ tissues in wild type and three independent mutant lines was analysed using Affymetrix Medicago GeneChips (Affymetrix, CA, USA). The ratio between mutant and wild type above twofold was considered up-regulated, while below 0.5 was considered down-regulated. We identified 826 up-regulated genes and 1216 down-regulated genes (Tables S3 and S4). The

differentially expressed genes were mapped in pathways including photosynthesis, hormone metabolism, signalling, stress, RNA, secondary metabolism, mitochondrial electron transport and DNA pathway (Figure S4a). Genes mapped in the hormone metabolism and cell wall pathways are shown in Figure S4b,c. In the hormone metabolism pathway, 67 auxin-related genes were down-regulated, including 53 SMALL AUXIN-UP RNA (SAUR) like family genes, 4 Gretchen Hagen3 (GH3) genes and 1 AUX/IAA gene. For ethylene-related genes, 3 ethylene response factors (ERFs), 3 1-aminocyclopropane-1-carboxylic acid synthases (ACSs) and the ethylene-insensitive 4 (EIN4) were down-regulated in the *plp* mutant (Figure S4b). In the cell wall pathway, the expression of 64 cell wall-related genes were significantly altered in *plp* mutant, including genes for polygalacturonase (PG), xyloglucan endotransglucosylase/hydrolase (XTH), glycosyl hydrolase (GH), pectin methylesterase (PME) and expansin (EXP) (Figure S4b). Twenty auxin-related genes were selected for PCR validation (Table S5), and they showed the same expression pattern as microarray data (Figure S5).

Microarray analysis revealed 152 putative abscission-related transcription factors (TFs). They belong to 23 different families of



**Figure 4** Anatomical comparisons between leaflet abscission zone (LAZ) and petiole abscission zone (PAZ) in wild type and *p/p*. (a, b) Close-up view of leaflet pulvinus region. (c–f) Microscopic analysis of longitudinal sections across the LAZ stained by toluidine blue at leaflet pulvinus region. (c, d) Mature leaflet (25-day-old) pulvinus region in wild type and *p/p*. No leaflet AZ was formed in either wild type or *p/p* mutant. (e, f) Senescing leaflet (35-day-old) pulvinus region in wild type and *p/p*. LAZ of wild type is shown in the picture, and no LAZ is formed in *p/p* mutant. (g, h) Close-up view of petiole pulvini region. (i–l) Microscopic analysis of longitudinal sections across the PAZ stained by toluidine blue at the petiole pulvinus region. (i, j) Mature leaf (25-day-old) petiole pulvinus region in wild type and *p/p*. No AZ was formed in either wild type or *p/p*. (k, l) Senescing leaf (35-day-old) petiole pulvinus region in wild type and *p/p*. Small cytoplasmic cells were observed along the petiole AZ region in wild type, and no AZ cells were observed in *p/p*. D25, 25-day-old leaf; D35, 35-day-old leaf. Red arrows indicate LAZ and PAZ; red asterisks in (f) and (l) indicate the region where the LAZ and PAZ should be present. Scale bars: (a,b) 5 mm; (g,h) 1 mm; (c–f,i–l) 500 μm. L, leaflet; PE, petiolule; PLP: petiolule-like pulvinus; PU, pulvinus; P, petiole; S, stem. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Figure 5** GUS staining of leaflet pulvinus region and petiole pulvinus region at different abscission zone developmental stages of transgenic *M. truncatula* plants carrying the *PLP* promoter-GUS construct. (a, e) GUS expression in the leaflet and petiole pulvinus region. (b, f) Prior to organ abscission, GUS expression was detected at the base of leaflet pulvinus and at the base of leaf pulvinus in 25-day-old leaf. (c, g) During organ abscission, GUS activity was detected at the base of 35-day-old leaflet and 43-day-old leaf petiole. (d, h) After abscission, no GUS expression was observed at the base of leaflet pulvinus or petiole pulvinus after the abscission of leaflet and petiole. L, leaflet; PE, petiolule; PU, pulvinus; P, petiole; S, stem; LAZ, leaflet abscission zone; PAZ, petiole abscission zone. Scale bars: (a, e) 5 mm; (b–d, f–h) 1 mm. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



TFs mainly including bHLH (8.6%), WRKY (8.6%), zinc fingers (8.6%), Aux/IAA family (7.9%), MYB (7.9%) and Homeobox (6.6%), suggesting a complex regulation of leaflet abscission in *M. truncatula* (Figure S6). In addition, there were several hormone-related TFs represented by AUX/IAA (7.9%), ARR (4.6%) and ARF (4.6%). We performed a Venn diagram analysis using our identified 152 putative abscission-related TFs and the 188 soybean abscission-specific TFs selected by Kim (Kim *et al.*, 2016). Totally 26 overlapped candidates are potentially involved in both soybean and *M. truncatula* leaf abscission (Figure S7; Table S6). These results suggest that *plp* affects auxin-related genes which, in turn, affect LAZ development in *M. truncatula*.

### Expression of the *NOOT* gene is down-regulated in *plp*

In order to examine whether there are any overlaps between published AZ-related genes and our microarray results, homologous genes of published genes related to AZ development were identified in *M. truncatula* by blast on phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) (Table S7). Except for the *NOOT* gene, none of the genes listed in Table S7 were significantly up- or down-regulated, indicating the regulatory pathway of floral organ abscission in *Arabidopsis* might be different from that of leaflet and petiole abscission in *M. truncatula*.

We investigated the relationship between *PLP* and *NOOT*. The *NOOT* gene is involved in leaflet, petiole, petal and seed abscission (Cougizou *et al.*, 2016). For wild type, a 39-day-old leaflet was already abscised from petiolule, while a 39-day-old leaflet of *noot* mutant was still attached to petiolule (Figure S8a, b). The 47-day-old petiole of wild type had already abscised from stem, while the petiole of *plp* was still attached to stem tightly (Figure S8a, b). The expression analysis of *NOOT* in wild type and *plp* mutants showed that the *NOOT* gene was significantly down-regulated for more than twofold compared with wild type (Figure S8e).

### Isolation and suppression of *MsPLP* gene in alfalfa

Because of the high degree of sequence identity between *M. truncatula* and alfalfa, the alfalfa *PLP* (*MsPLP*) gene was cloned using the primers derived from *MtPLP*. Sequence comparison analysis showed that *MtPLP* and *MsPLP* share 97.4% similarity (Figure S9). Analysis of deduced amino acid sequences revealed that *MsPLP* protein contained 193 amino acids, showing 99% identity to *MtPLP* (Figure S10). The *MsPLP* protein is also highly similar to its putative orthologs in *Glycin max*, *Lotus japonicas*, *Pisum sativum* and *Trifolium medium*, indicating *PLP* is highly conserved in legume species (Figure S10). The *PLP* orthologs in various species were obtained by BLASTN through NCBI and Phytozome and used for phylogenetic analysis (Figure S11). Phylogenetic analysis showed that *MsPLP* and *MtPLP* were closest to each other and were clustered close to *PsAPU*, *TmELP1* and *TpLOB* (Figure S11).

To suppress the activity of the endogenous *MsPLP* in alfalfa, *MsPLP*-RNAi vectors (Figure S12) were constructed and introduced into alfalfa by *Agrobacterium tumefaciens*-mediated transformation (Figure S13a-e). Only one plant was selected from each callus, representing an independent line. Fourteen independent transgenic lines were identified by PCR analysis (Figure S13f). Quantitative RT-PCR analysis revealed that 12 transgenic lines (S1-S12) had *MsPLP* expression levels reduced by more than 75% when compared with the wild type (Figure S14a). Three transgenic lines, S1, S2 and S3, were used for further analysis.

### *MsPLP*-RNAi transgenic alfalfa shows defects in pulvinus development

Plant height and fresh weight showed no significant differences between wild type and transgenic lines, suggesting that knocking down *MsPLP* had no effects on biomass yield (Figure S14b-d). The RNAi alfalfa plants showed defects in leaflet and petiole pulvinus development (Figure 6a-h) similar to the *M. truncatula plp* mutant.

### Suppression of *MsPLP* strengthens the leaflet and petiole pulvinus region

Leaflet and petiole breakstrengths were measured when plants reached blooming stage, at which alfalfa is normally harvested. In the field, alfalfa plants are generally harvested 10 cm above ground. Therefore, we evaluated the leaflets above the height of 10 cm. Breakstrengths of 3<sup>rd</sup> leaflets to 12<sup>th</sup> leaflets were evaluated (Figure 7a). For both terminal and lateral leaflets, leaflet breakstrengths of transgenic lines were significantly higher than that of wild type (Figure 7b, c). For petiole breakstrength, 3<sup>rd</sup> to 5<sup>th</sup> petiole and 11<sup>th</sup> to 12<sup>th</sup> petiole of the transgenic lines were significantly higher than that of wild type (Figure 7d).

In order to further characterize whether the pulvinus region was strengthened, the frequency of the breaking positions during breakstrength assay was recorded. In wild type, the terminal leaflet, lateral leaflet and petiole were detached specifically at the pulvinus region during the breakstrength assay, indicating the pulvinus region is the weakness area (Figure S15a, c). In transgenic lines, the detachment of terminal leaflet occurred at three positions, D1: leaflet (8%), D2: petiolule-like pulvinus (22%) and D3: petiolule (70%); the detachment of lateral leaflet occurred at three positions, D1: leaflet (2%), D2: petiolule-like pulvinus (57%) and D3: base of petiolule-like pulvinus (41%); and the detachment of petiole occurred at two positions, D1: petiole-like pulvinus (95%) and D2: petiole (5%) (Figure S15b, c). The various detachment position demonstrates that the pulvinus region was strengthened by genetically knocking down the *PLP* gene in alfalfa.

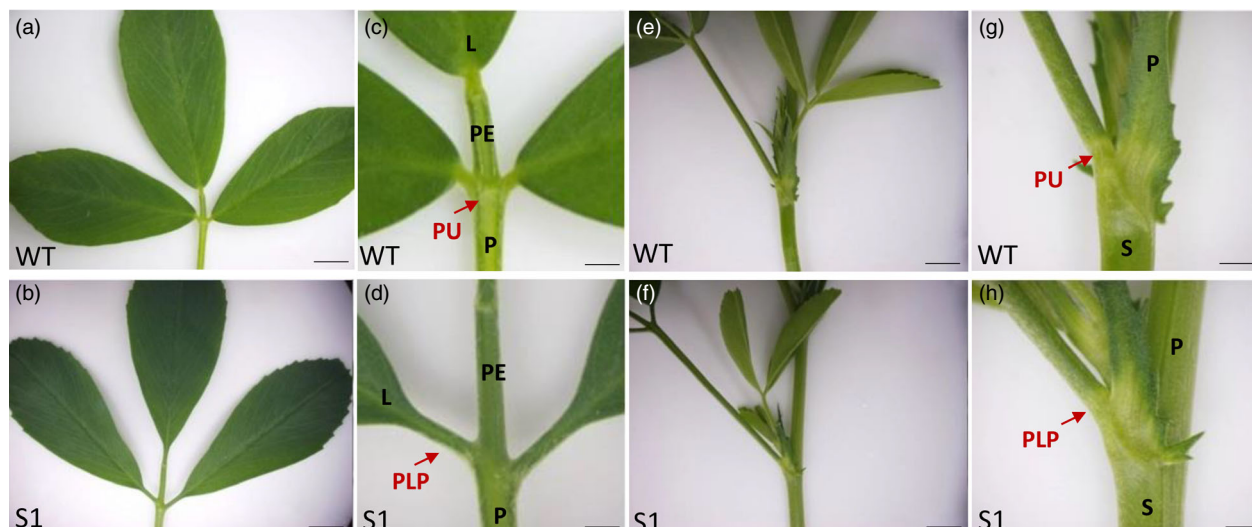
### Analysis of nutritive value of *MsPLP* down-regulated alfalfa plants

In order to evaluate whether the knockdown of *MsPLP* will affect alfalfa quality under greenhouse non-senescing conditions, acid detergent fibre (ADF), neutral detergent fibre (NDF), crude protein (CP), in vitro true dry matter digestibility (IVTDM), total digestible nutrients (TDN) and relative feed value (RFV) were analysed. No significant change was found in these forage quality parameters between wild type and transgenic lines for the whole plant, leaf and stem (Figure S16a-c). The results show that down-regulation of *MsPLP* has no impact on alfalfa nutritive value when the vegetative plants were gently harvested without leaf loss.

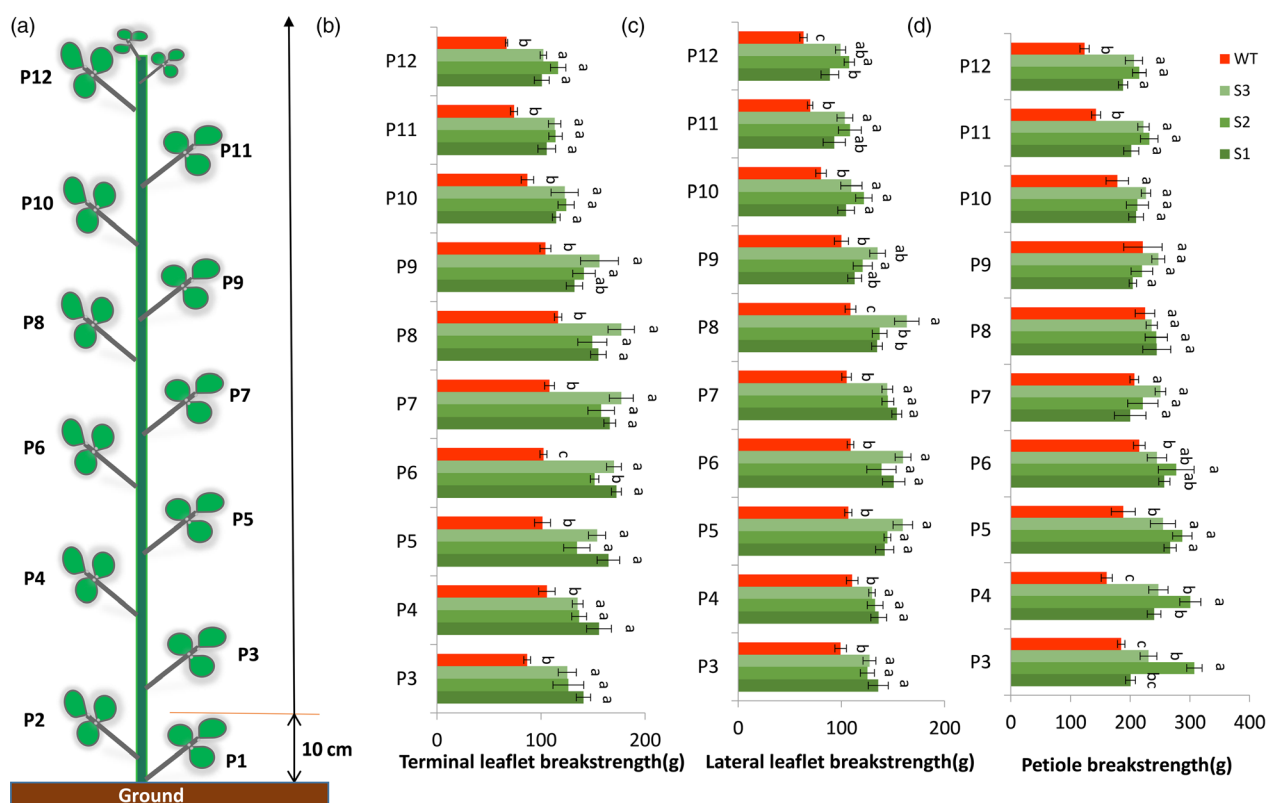
## Discussion

### *PLP* is required for AZ differentiation

Abscission zone is a unique anatomical structure which is essential for the abscission process. Several genes involved in *Arabidopsis* floral organ (*BOP1/BOP2*, *ATH1* and *AS1*), tomato pedicel (*JOINTLESS*, *MC* and *SLMBP21*) and rice pedicel (*SH4*, *QSH1*, *SHAT1*, *OsSH1* and *OsCPL1*) abscission zone differentiation have been identified (Gómez-Mena and Sablowski, 2008; Gubert *et al.*, 2014; Ji *et al.*, 2010; Konishi *et al.*, 2006; Li *et al.*,



**Figure 6** *MsPLP*-RNAi transgenic lines showed defects in pulvinus development. (a, b) Leaf of wild type and *MsPLP*-RNAi alfalfa. (c, d) Close-up view of leaflet base in wild type and transgenic alfalfa. Transgenic alfalfa showed defects in pulvinus development, pulvinus was altered to petiolule-like structure. (e, f) Petiole of wild type and *MsPLP*-RNAi alfalfa. (g, h) Close-up view of petiole base in wild type and transgenic alfalfa. Transgenic alfalfa showed defects in petiole pulvinus development. L, leaflet; PE, petiolule; PU, pulvinus; P, petiole; S, stem; PLP, petiolule-like pulvinus. Scale bars: (a, b) 1 cm; (c, d) 1 mm; (e, f) 1 cm; (h, 1) 1 mm. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Figure 7** Terminal and lateral leaflet breakstrength assay and petiole breakstrength assay of wild type and transgenic alfalfa. (a) Schematic leaflet distribution on alfalfa stem. The evaluation of leaflet and petiole breakstrength was started from the third leaflet because alfalfa is normally harvested 10 cm above ground under field conditions. (b) Terminal leaflet breakstrength measurements at blooming stage. (c) Lateral leaflet breakstrength measurements at blooming stage. (d) Petiole breakstrength measurements at blooming stage. Error bars indicate SD ( $n = 5$ ). P3, the 3rd leaflet; P4, the 4th leaflet; ... P12, 12th leaflet. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

2006; Lin *et al.*, 2012; Liu *et al.*, 2014; Mao *et al.*, 2000; McKim *et al.*, 2008; Nakano *et al.*, 2012; Zhou *et al.*, 2012b). In *M. truncatula*, the *NOOT* gene, a *BOP* ortholog, has been shown to modulate petal, leaflet and seed abscission zone differentiation (Couzigou *et al.*, 2016). Interestingly, in both *plp* and *noot* mutants, simplified stipules were observed, while the pulvinus structure of *noot* mutant is not modified as the *plp* mutant, suggesting that the absence of *PLP* activity modifies the cellular structure of the tissue where leaflet and petiole AZs are differentiated. Until now, the molecular mechanism of leaflet and petiole AZ differentiation is still largely unknown. The present study focused on leaflet and petiole abscission. We discovered that the *PLP* gene plays an important role in regulating leaflet and petiole abscission zone differentiation in *M. truncatula*. The loss of function of *PLP* results in a complete absence of leaflet and petiole abscission during plant senescence. Microscopic observation of cross sections and SEM analysis further confirmed that the *plp* mutants have no leaflet and petiole abscission zone differentiation, suggesting the importance of *PLP* in initiating AZ differentiation.

#### Lateral organ boundary formation and abscission zone development

Night closure of leaflets is a common feature of legumes, and such nyctinastic movement is generated by the pulvinus. It has been proposed that *PLP* regulates pulvinus establishment through controlling boundary formation for the correct production of compact and convoluted motor cells to mediate leaflet movement (Zhou *et al.*, 2012a). *PLP* is a LOB domain protein, and it has 75% identity to *Arabidopsis ASL4/LOB* which belongs to the LBD protein family (Zhou *et al.*, 2012a). LBD proteins play important roles in modulating plant development (pollen, embryo, root, leaf and inflorescence development), hormone response (cytokine, ABA, auxin, JA, gibberellin), anthocyanin and nitrogen metabolism, plant regeneration (callus formation), disease susceptibility, photomorphogenesis, secondary growth and pulvinus development (Majer and Hochholdinger, 2011; Xu *et al.*, 2016). LBDs are specifically expressed at organ boundaries regulating plant lateral organ development (Yang *et al.*, 2016). In wild type, there is a clear boundary region at the base of leaflet and petiole pulvinus, whereas no boundary was formed at the base of leaflet and petiole in the *plp* mutant (Figure 4). The wild-type leaflet and petiole AZs were differentiated after the development of leaflet and petiole pulvinus (Figure 4c,e,i,j); thus, they are adventitious AZs according to Addicott (1982). In the *plp* mutant, the loss of cell fate in leaflet and petiole AZs led to the failure of AZ cell placement and differentiation. These observations indicate that the existence of organ boundaries influences AZ formation. Several genes that are involved in organ boundary formation have been shown to be involved in AZ development including *BOP1/2*, *ATH1*, *AS1* and *HWS* (Gómez-Mena and Sablowski, 2008; González-Carranza *et al.*, 2007; Gubert *et al.*, 2014; Hepworth *et al.*, 2005; Jun *et al.*, 2010; McKim *et al.*, 2008; Norberg *et al.*, 2005; Xu *et al.*, 2008). Our study provides more evidence to support the potential link between organ boundary formation and AZ development.

In *Arabidopsis*, *BOP1/BOP2* have been demonstrated as the earliest regulators associated with AZ differentiation, which are required for abscission in both wild-type and *35S::IDA* plants (McKim *et al.*, 2008). The homologous genes of *BOP* in tobacco (*Nicotiana tabacum*) as well as in a few legume species (*Pisum*

*sativum*, *Lotus japonicas*, *Lupinus angustifolius* and *Lupinus luteus*) are also necessary for the abscission of lateral organs (Couzigou *et al.*, 2016; Frankowski *et al.*, 2015; Kućko *et al.*, 2019; Wu *et al.*, 2012). In *M. truncatula*, the *noot* mutant has no specialized small condensed cell layer along its leaflet and leaf abscission zone region, indicating the role of *NOOT* gene in regulating the establishment of AZ (Couzigou *et al.*, 2016). In the current study, we found that the *NOOT* gene was down-regulated in the *plp* mutant, suggesting a possible relationship between *PLP* and *NOOT*. It has been shown that the LOB domain genes *ASYMMETRIC LEAVES2* and *LOB* were up-regulated in *35S::BOP* and down-regulated in *bop1/2* mutant, therefore indicating a potential link between these genes in regulating lateral organ cell fate and polarity in *Arabidopsis* (Ha *et al.*, 2007). In addition, the overexpression of *LBD15* activated the transcription of *BOP1* and *BOP2*, which were critical in controlling lateral organ development and inflorescence architecture (Zhu *et al.*, 2014). Since *PLP* is an LBD protein, we propose a potential link between *PLP* and *NOOT* in defining the AZ cells at organ boundaries by modulating lateral organ polarity and cell fate. Further research on genetic interactions between *PLP* and *NOOT* may provide more details about AZ differentiation.

#### Transcriptional regulation of hormonal homeostasis, cell wall degradation and remodelling by *PLP* in the abscission process

The conventional model suggests that auxin gradient plays a central role in abscission (Addicott, 1982). The sensitivity of AZ to ethylene is regulated by the alteration of auxin gradient (Abeles and Rubinstein, 1964; Meir *et al.*, 2006). Previous research on *PLP* used a DR5rev: GFP auxin response reporter to detect the role of *PLP* in auxin signalling (Zhou *et al.*, 2012a). A GFP signal was detected specifically in the wild-type pulvini, while no signal was observed in the *plp-1* mutant (Zhou *et al.*, 2012a). This result was used to confirm the alteration of the unique structure of pulvini in the mutant without realizing its implications in abscission (Zhou *et al.*, 2012a). Here, when abscission is considered, the result also suggests that auxin gradient across AZ was affected due to *PLP* mutation. Our microarray results showed that 137 genes involved in hormone metabolism were affected by the mutation of *PLP*. Among them, 70 auxin-related genes and 12 ethylene-related genes were significantly affected, indicating a possible role of *PLP* in modulating abscission process through auxin and ethylene biosynthesis and signalling.

Auxin response factors (ARFs) are transcription factors that mediate responses to auxin. In *Arabidopsis*, *ARF2* was reported to regulate floral organ abscission (Ellis *et al.*, 2005). *ARF1* or *NPH4/ARF7* and *ARF19* were reported to enhance the delayed abscission of *arf2* mutant (Ellis *et al.*, 2005; Okushima *et al.*, 2005). The tomato homologue of the *Arabidopsis ARF19* was down-regulated in *KD1* antisense transgenic plants with delayed pedicel abscission phenotype (Ma *et al.*, 2015). In tomato, *miR160* was found to regulate floral organ abscission and fruit abscission (Damodharan *et al.*, 2016). The sly-miR160 depletion was associated with the up-regulation of ARFs including *SIARF10A*, *SIARF10B* and *SIARF17* which are homologues of *Arabidopsis ARF10* and *ARF17* (Damodharan *et al.*, 2016). The expression pattern of ARFs during tomato flower pedicel abscission under auxin and ethylene treatment suggested important roles of ARFs in regulating pedicel abscission (Guan *et al.*, 2014). Studies on *LBD18*, *LBD16* and *LBD30* revealed the function of LBDs in

regulating auxin signalling pathway. *LBD18* functions in the initiation and emergence of lateral roots, in conjunction with *LBD16*, downstream of *ARF7* and *ARF19* (Lee et al., 2009). *JLO* (*LBD30*) is expressed in boundaries and regulates both auxin transport and meristem fate by promoting the expression of the *KNOX* genes *SHOOTMERISTEMLESS* (*STM*) and *BPIKNAT1* (Bureau and Simon, 2008). In our study, eight *ARFs* were affected in *plp* mutant, including *ARF8*, *ARF10*, *ARF16* and *ARF19*, indicating a possible role of *PLP* in regulating auxin response to control the abscission process.

Abscission is activated after perceiving abscission signals, such as auxin and ethylene in the AZ. The microarray analyses of 25-day-old leaflet abscission zone region showed that several genes for cell wall degrading and remodelling factors were down-regulated in the *plp* mutant. These include PGs, XTHs, GHs, PMEs and EXPs. The roles of these cell wall modifying enzymes have been associated with abscission in *Arabidopsis* and tomato (Estornell et al., 2013; Hong et al., 2000; Jiang et al., 2008; Lashbrook and Cai, 2008; Ogawa et al., 2009). In our study, abscission was not yet detected in 25-day-old leaflets (Table S1). The expression of *PLP* gene was detected in both prior and during the abscission process in Figure 5(a-c, e-g), suggesting that *PLP* might play a role not only in AZ differentiation but also in the competence to respond to abscission signals and the activation of abscission. Both *HAE* and *HSL2* regulate programmed separation of cells during abscission (Cho et al., 2008). Interestingly, *AtLOB*, the homologous gene of *MtPLP* in *Arabidopsis*, is up-regulated in floral receptacles of the *Arabidopsis* double mutant *hae hsl2*, indicating that the *AtLOB* is closely related to abscission execution (Niederhuth et al., 2013). This reinforces the regulatory role of *MtPLP* on hormonal- and cell wall-related genes during leaflet abscission. Taken together, the above evidence consistently suggests that *PLP* affects abscission execution through transcriptional regulation of hormonal homeostasis, cell wall degradation and remodelling. Here we propose a model of *PLP* in regulating leaflet and petiole abscission (Figure S17).

### Promising candidate genes in leaf abscission studies

To analyse genes involved in leaf abscission in soybean, Kim et al. (2016) performed RNA sequencing (RNA-seq) using RNA isolated from the leaf abscission zones and petioles (Non-AZ, NAZ) of soybean after treating stem/petiole explants with ethylene for 0, 12, 24, 48 and 72 h. 188 abscission-specific TFs were identified, which include TFs belonging to homeobox, MYB, Zinc finger, bHLH, AP2, NAC, WRKY, YABBY and ARF families (Kim et al., 2016). In the present study, we identified 26 overlapped candidates that are involved in both soybean and *M. truncatula* leaf abscission (Figure S7). These genes are promising candidates for future leaf abscission studies (Table S6).

### *PLP* has potential for alfalfa improvement

In the present study, we explored a new way to minimize leaf loss during the haymaking process through genetically modifying alfalfa leaflet and petiole structures. The leaflet pulvinus and petiole pulvinus regions were strengthened with increased breakstrength and decreased detachment frequency by RNAi suppression of *MsPLP* without negative impacts on biomass yield and nutritive value. The abscission of leaflet and petiole was abolished in transgenic alfalfa. In future studies, a complete knockout of *PLP* in alfalfa can be achieved by genome editing (Wolabu et al., 2020). A large-scale field test is needed to

evaluate leaf loss and forage quality of the materials using commercial machines under field conditions.

In summary, our study revealed a new function of *PLP* in regulating AZ development in the model legume *M. truncatula* and we have successfully applied this knowledge to alfalfa improvement. This study demonstrated that loss of function of *PLP* resulted in an absence of AZ differentiation. The regulation of abscission by *PLP* is associated with auxin-related genes and *NOOT*. Furthermore, the alteration of pulvinus to petiole-like pulvinus has significantly increased the breakstrength of leaflets and petioles in alfalfa, which offers a new approach to decrease leaf loss during the haymaking process. This study demonstrates how knowledge gained from a model plant can be applied to the genetic improvement of a commercial crop.

## Experimental procedures

### Plant materials and growth conditions

The *Medicago truncatula* Gaertn. Ecotype R108 was used as the wild type in this study. The source of the three *plp* alleles (Zhou et al., 2012a) and the *noot* allele (Couzigou et al., 2016) is described in Table S8. Mutant and wild-type seeds were scarified with sandpaper and treated at 4°C for 7 days on filter paper and then transferred into soil. An alfalfa genotype, Regen SY-4D, was used for *Agrobacterium tumefaciens*-mediated transformation to produce transgenic plants following the protocol described by Fu et al. (2015). All the plants were grown in Metro-Mix 830 soil in the greenhouse with the condition 22°C day/20°C night temperature, 16-h light/8-h dark photoperiod and 70-80% relative humidity.

### Isolation of *MsPLP* and creation of *MsPLP* modified transgenic alfalfa plants

The alfalfa *PLP* (*MsPLP*) gene was cloned using the following primers designed based on *MtPLP*: MsPLPFLF2: ATTGCTTTGTTG-CAGGAGAA and MsPLPFLR2: CAAGAGACATAACATAAA-TAAACCCT. To knockdown the expression of *MsPLP* in alfalfa, 262-bp and 205-bp *MsPLP* fragments were amplified from alfalfa (genotype Regen SY-4D) cDNA using primers shown in Table S9. Each fragment was independently cloned into the PENTR™/D-TOPO cloning vector (Invitrogen, Chicago, IL, USA) and transferred into the pANDA35HK vector (Miki & Shimamoto, 2004) by attL × attR recombination reactions (Invitrogen). Sonication-assisted *Agrobacterium*-mediated transformation and tissue culture were performed for transgenic alfalfa generation (Fu et al., 2015). PCR analysis of the regenerated plants was performed using primers from the GUS linker of the pANDA35HK vector: GUS4F: CATGAAGATGCGGACTTACG and GUS5R: ATC-CACGCCGTATTCCG.

### Protein alignment, phylogenetic analysis and expression pattern of *MtPLP*

Predicted PLP amino acid sequences from various plant species were obtained by protein blast in GenBank (<http://www.ncbi.nlm.nih.gov/>) or Phytozome (<https://phytozome.jgi.doe.gov/>). All sequences were subsequently aligned using the ClusterW by DNASTAR. A phylogenetic tree was built using the neighbour-joining method by employing the DNASTAR software. The DNA sequence of *MtPLP* was used for the analysis of expression pattern through BLAST function in Gene Expression Atlas (<https://mtgea.noble.org/v2/>).

### Microscopic examination of leaflet and petiole abscission zone

Leaflet pulvinus region and petiole pulvinus region of *M. truncatula* mutants and transgenic alfalfa were carefully examined under a dissecting microscope.

In *M. truncatula*, 25-day-old leaves (mature leaves) represent the stage that prior to leaflet abscission and petiole abscission. Thirty five-day-old leaves (senescing leaves) represent the stage when leaflet and petiole are under the abscission process. The 25-day-old and 35-day-old AZ regions of petiole and leaflet were collected and fixed with 4% paraformaldehyde, 2.5% glutaraldehyde and 0.01% (v/v) Triton X-100 dissolved in PBS (pH7.4) at 4°C for overnight. All specimens were then dehydrated in a series of ethanol and embedded in LR White resin (London Resin Co. Ltd., Berkshire, UK) using gelatin capsules. The resin was polymerized at 60°C for two days. In order to closely examine the abscission zones from wild type and *plp* mutant, serial 0.25-micrometre longitudinal sections were cut with a diamond knife with a Leica EM UC7 ultramicrotome (Leica Microsystems GmbH, Vienna, Austria) or RMC MT-X ultramicrotome (Boeckeler Instruments, Inc., Tucson, Arizona, USA). Semi-thin sections (0.25 µm in thickness) placed onto the slide glasses (Superfrost Plus; VWR International, LLC, Radnor, Pennsylvania, USA) were stained with 1% (w/v) Toluidine Blue O (with 1% (w/v) sodium borate) for 5 min and observed under a Nikon Microphot-2 (Nikon Corporation, Tokyo, Japan).

For Zeiss NEON 40 EsB scanning electron microscopy (SEM) examination of leaflet and petiole AZs, leaflets and petioles (31, 35, 39, 43 and 47 days old) were removed at the pulvinus if not yet abscised from the plant in wild type and *plp* mutant. Samples were fixed, dried and sputter-coated as reported previously (Gou *et al.*, 2017). The fracture planes on petiolule and stem created by removing leaflets and petiole or naturally abscised were observed.

### Shaking assay for abscission

*M. truncatula* plants were staked in a similar manner in the greenhouse. Both mutant and wild-type plants at senescence stage (108-day-old plant) were shaken vigorously for 2 min by hand. The leaves dropped onto ground were collected and weighed.

### Breakstrength assay

Leaflet breakstrength is the force in gram equivalents required to remove a leaflet from the petiolule. Petiole breakstrength is the force to remove a whole leaf from the stem. The leaflet and petiole breakstrengths were measured by using the CT3 Texture Analyzer (BROOKFIELD, MA) in tension mode. Samples were clapped at the same position with dual grip assembly (TA-DGF001) and extended at a constant speed of 2.0 mm s<sup>-1</sup> until breakage and the breakstrength was recorded. For both wild type and the *plp* mutants in *M. truncatula*, we started to mark fully expanded leaves as one-day-old leaves when plants were 48 days old. Afterwards, leaf samples at different ages from 25 to 49 days were collected in two-day intervals for leaflet and petiole breakstrength measurements (eight replicates). For alfalfa materials, we evaluated the terminal, lateral leaflet and petiole breakstrengths from the 3<sup>rd</sup> to 12th leaflets, which represent leaflets that are normally harvested during alfalfa haymaking process. The breakstrength data were analysed by SPSS software.

### β-Glucuronidase (GUS) staining

To determine whether there is a correlation between the *PLP* expression pattern and leaflet and petiole abscission, we examined transgenic plants that express β-glucuronidase (GUS) under the control of *PLP* promoter. GUS expression was analysed during the time course of abscission in three stages as shown in Table S2: stage I, prior to organ separation; stage II, during abscission; and stage III, after abscission when the remaining cells form a protective scar tissue. At stage II, the organs were separated manually.

### RNA extraction, qRT-PCR and microarray analysis

Pulvinus regions (abscission zones included) of 25-day-old leaflets from wild type and three *plp* alleles of *M. truncatula* (85-day-old plants) and of wild type and 14 transgenic alfalfa lines were collected for RNA isolation, cDNA reverse transcription and qRT-PCR as previous described (Gou *et al.*, 2019; Zhou *et al.*, 2012a). Primers for quantifying the expression levels of auxin-related genes and the *NOOT* gene are listed in Table S10.

For microarray analysis, 10 µg of purified RNA was extracted from the pulvinus region of 25-day-old leaves of *Medicago truncatula*. RNA purification, probe labelling, hybridization, scanning and microarray data analysis were performed following the manufacturer's instructions (Affymetrix; <http://www.affymetrix.com>). Functional enrichments were visualized with MAPMAN (Thimm *et al.*, 2004).

### Forage analysis of transgenic alfalfa lines

Three transgenic alfalfa lines S1, S2, S3 and control wild-type alfalfa plants were grown in the greenhouse and propagated by cuttings. At early blooming stage, plants were harvested and fresh biomass yield was measured immediately. Forage quality was analysed using near-infrared reflectance spectroscopy (NIRS) as previous described (Gou *et al.*, 2018).

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### Conflicts of interest

The authors declare no conflict of interest.

### Author contribution

ZYW, YZ and JD designed and planned the experiments; JD, SL, MC, CZ, JN, JK, ZW, MB, JS and TZ performed research; JD, YT and LS analysed the data; and JD, YZ and ZYW wrote the manuscript.



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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Petiole-like pulvinus (*plp*) mutant of *M. truncatula* shows developmental defects in leaflet and petiole pulvini.

**Figure S2** Leaf dropping after shaking assay in *plp* mutant of *M. truncatula*.

**Figure S3** Expression pattern of *MtPLP* in wild type, generated from the *M. truncatula* Gene Expression Atlas.

**Figure S4** Differential expression of genes in pulvinus region of 25-day-old leaves of wild type and *plp* mutant of *M. truncatula*.

**Figure S5** Validation of microarray data by qPCR of auxin-related genes in AZ region of *M. truncatula*.

**Figure S6** Pie chart of leaflet abscission-related transcription factors (TFs) differentially expressed in *M. truncatula* during leaflet abscission.

**Figure S7** Venn diagram analysis of the overlap of abscission-specific TFs in soybean and TFs that are differentially expressed in *plp* mutant of *M. truncatula*.

**Figure S8** Loss of leaflet and petiole abscission phenotype in *noot* mutant and the expression of *NOOT* in wild type and *plp*.

**Figure S9** Sequence comparison of *MsPLP* (*M. sativa* SY4D) with *PLP* (*M. truncatula* R108).

**Figure S10** Amino acid alignment of *MsPLP* (*M. sativa* SY4D) with *PLP* (*M. truncatula* R108) and their orthologs from *Arabidopsis thaliana* (At), *Glycine max* (Gm), *Lotus japonicas* (Lj), *Pisum sativum* (Ps) and *Trifolium medium* (Tm).

**Figure S11** Phylogenetic analysis of PLP-related orthologs in different plant species.

**Figure S12** Construction of *MsPLP*-RNAi binary vectors for alfalfa transformation.

**Figure S13** Generation of *MsPLP*-RNAi transgenic alfalfa plants.

**Figure S14** Molecular and phenotypic characterization of alfalfa *MsPLP*-RNAi transgenic lines.

**Figure S15** Frequency of detachment occurs at marked positions in both wild type and transgenic alfalfa lines.

**Figure S16** Evaluation of nutritive quality of transgenic alfalfa lines.

**Figure S17** A proposed model of *PLP* in regulating leaflet and petiole abscission.

**Table S1** Characterization of leaflet and petiole abscission in wild type and *plp* mutant of *M. truncatula*.

**Table S2** Abscission stages of leaflet and petiole according to leaf ages.

**Table S3** List of genes that are up-regulated in *plp* mutant.

**Table S4** List of genes that are down-regulated in *plp* mutant.

**Table S5** List of auxin-related genes that were down-regulated in LAZ for qPCR validation.

**Table S6** List of transcription factors in *M. truncatula* that overlapped with abscission-specific TFs in soybean.

**Table S7** List of homologous genes of already published genes related to AZ development in *M. truncatula*.

**Table S8** List of *M. truncatula* lines used in this study.

**Table S9** List of primers used for *MsPLP*-RNAi vector construction.

**Table S10** Primers for qPCR analysis of auxin-related genes and the *NOOT* gene.